12 OPIOID QUANTITATION AND CONFIRMATION BY GCMS

12.1 Summary

12.1.1 Opioid drugs are extracted from biological samples using an acetonitrile precipitation, solid phase extraction (SPE) and analyzed by selected ion monitoring GCMS. Morphine, codeine, 6-acetylmorphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, and deuterated internal standards are extracted and analyzed simultaneously. The keto opioids are reacted with hydroxylamine to form oxime derivatives to prevent tautomerism. Hydroxyl groups of all opioids are further derivatized with BSTFA with 1% TMCS to form trimethylsilyl derivatives. The high mass compounds formed enable chromatographic separation and quantitation of all seven opioids simultaneously. This procedure presents two options for extraction.

12.2 Specimen Requirements

12.2.1 Two mL blood, urine, vitreous humor, gastric or tissue homogenate

12.3 Standards and Reagents

12.3.1 Standards

12.3.1.1 6-acetylmorphine, 1 mg/mL

12.3.1.2 6-acetylmorphine-d_3, 100 μg/mL

12.3.1.3 Codeine, 1 mg/mL

12.3.1.4 Codeine-d_3, 1 mg/mL

12.3.1.5 Morphine, 1 mg/mL

12.3.1.6 Morphine-d_3, 1 mg/mL

12.3.1.7 Hydrocodone, 1 mg/mL

12.3.1.8 Hydrocodone-d_3, 100 μg/mL

12.3.1.9 Hydromorphone, 1 mg/mL

12.3.1.10 Hydromorphone-d_3, 100 μg/mL

12.3.1.11 Oxycodone, 1 mg/mL

12.3.1.12 Oxycodone-d_3, 1 mg/mL

12.3.1.13 Oxymorphone, 1 mg/mL

12.3.1.14 Oxymorphone-d_3, 100 μg/mL

12.3.2 Reagents.

12.3.2.1 Ammonium hydroxide

12.3.2.2 Acetic acid, glacial

12.3.2.3 Hydrochloric Acid, concentrated

12.3.2.4 Potassium Hydroxide

12.3.2.5 Potassium Phosphate

12.3.2.6 BSTFA containing 1% TMCS

12.3.2.7 Ethyl Acetate

12.3.2.8 Methanol

12.3.2.9 Acetonitrile

12.3.2.10 Hydroxylamine Hydrochloride

12.3.2.11 Hexane

12.3.2.12 Sodium acetate trihydrate

12.3.2.13 Methylene chloride

12.3.2.14 Isopropanol

12.3.2.15 Ammonium hydroxide

12.4 Solutions, Internal Standards, Calibrators, Controls

12.4.1 Solutions

12.4.1.1 1 M Acetic Acid. Add 5.75 mL of glacial acetic acid to a 100 mL volumetric flask half filled with dH_2O. QS to volume with dH_2O.

12.4.1.2 5.0 M Potassium Hydroxide. Weigh 28 g of potassium hydroxide into a 100 mL volumetric flask. Dissolve the potassium hydroxide with dH_2O. QS to volume with dH_2O.

12.4.1.3 0.1 M Phosphate Buffer, pH 6.0. Weigh out 13.61 g of KH_2PO_4 and transfer into a 1 liter volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5.0 M potassium hydroxide while stirring. QS to volume with dH_2O.

12.4.1.4 2 % Ammonium Hydroxide in Ethyl Acetate. Pipette 2.0 mL of concentrated ammonium hydroxide into a 100 mL graduated cylinder filled with 98 mL of ethyl acetate. Cap graduated cylinder with a glass stopper, and mix well. Vent cylinder occasionally. PREPARE SOLUTION FRESH DAILY!

12.4.1.5 1% Hydroxylamine. Weigh 250 mg of hydroxylamine and add into a 25 mL volumetric flask. QS to volume with dH_2O.

12.4.1.6 100 mM acetate buffer (pH 4.5). Weigh 2.93 g sodium acetate trihydrate into a 500 mL volumetric flask. Add approximately 400 mL dH_2O to dissolve sodium acetate. Add 1.62 mL glacial acetic acid. Adjust pH to 4.5 with 1 M acetic acid. QS to volume with dH_2O.

12.4.1.7 Dichloromethane/isopropanol 80:20 v/v. Mix 800 mL dichloromethane with 200 mL isopropanol.

12.4.1.8 Dichloromethane/isopropranol/ammonium hydroxide elution solvent. Add 3 mL concentrated ammonium hydroxide to 100 mL dichloromethane/isopropanol 78:20 v/v. PREPARE SOLUTION FRESH DAILY!

12.4.2 Calibrators and internal standards. Calibrators may be prepared in pools and stored as frozen aliquots or as fresh spikes.

12.4.2.1 Pooled calibrator preparation. Calibrator working stock standard (10 / 2.5 µg/mL). Add 100 µL each of 1 mg/mL codeine, morphine, hydrocodone, hydromorphone, oxycodone and oxymorphone standards into a 10 mL volumetric flask. Add 25 µL of 1 mg/mL 6-acetylmorphine standard to the same 10 mL volumetric flask. QS to volume with distilled water. The working stock standard final concentration is 10 µg/mL codeine, morphine, hydrocodone, hydromorphone, oxycodone and oxymorphone and 2.5 µg/mL 6-acetylmorphine.

12.4.2.1.1 Preparation of calibrator blood stocks. Pipet the following volumes of working stock standard into 50 mL volumetric flasks. QS to volume with opioid-free blank blood. Once the accuracy and linearity are verified, aliquot 2 mL of calibration blood stocks into 16 x 125 mm screw top tubes and freeze for future use.

12.4.2.1.2

Blood Calibrator (µg/L) Opioid/6AM	Volume (µL) Working Stock Standard (10/2.5 µg/mL)
20 / 5	100
40 / 10	200
100 / 25	500
300 / 75	1500
500 / 125	2500
800 / 200	4000

12.4.2.2 Spiked calibrator preparation. Calibrator working stock standard (2.0 µg/mL). Add 50 µL each of 1 mg/mL codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone and 6-acetylmorphine standards into the same 25 mL volumetric flask. QS to volume with methanol. Other quantitative dilutions may be acceptable to achieve similar results.

12.4.2.2.1 Use the following table to prepare desired calibrators. Add calibrator working stock standard (2 µg/mL) to labeled tubes.

Blood Opioid Calibrator (µg/L)	Volume (µL) Working Stock Standard (2µg/mL)
10	10
20	20
50	50
100	100
200	200
400	400
600	600

12.4.2.3 Internal standards. Internal standard spiking stock. Pipet 25 µL of 1 mg/mL opioid deuterated internal standards and 250 µL of 100 µg/mL deuterated internal standards (except 6-acetylmorphine) to a 10 mL volumetric flask. Add 100 µL of 100 µg/mL 6-acetylmorphine-d_3 into the same volumetric flask. QS to

volume with methanol. Add 100 µL of internal standard spiking stock to 2 mL blank blood for final concentration of 50 µg/L for 6-acetylmorphine and 125 µg/L for all other deuterated opioids.

12.4.2.4 A single internal standard (listed in 12.3.1) may be used for more than one analyte.

12.4.3 Controls.

12.4.3.1 Negative blood control. Blood bank blood or equivalent previously determined not to contain opioids.

12.4.3.2 Positive blood control (100 µg/L codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone and 6-acetylmorphine). The positive control may be purchased or prepared in-house. Do not use calibrator working stock standard to prepare controls. The in-house positive control may be prepared as a pooled control or spike. Other positive control concentrations may be prepared.

12.4.3.2.1 Pooled control working stock standard (5 µg/mL codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone and 2.5 µg/mL 6-acetylmorphine). Pipet 50 µL each of 1 mg/mL codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone standards and 25 µL of 1 mg/mL 6-acetylmorphine standard into a 10 mL volumetric flask. QS to volume with dH_2O.

12.4.3.2.1.1 Pooled blood control. Pipet 1.0 mL of control working stock solution into a 50 mL volumetric flask. QS to volume with blank blood. Once concentration is verified, aliquot 2 mL of positive blood control into 16 x 125 mm screw top tubes and freeze for future use.

12.4.3.2.2 Spiked control. From a separate pipeting, prepare the control spiking stock as per 12.4.2.1. Add 50 µL to a test tube. Evaporate methanol, add 2 mL blank blood and vortex. Different concentrations and preparations of spiked controls are permissible.

12.4.3.2.3 Commercial control. QAS or other commercial vendors. Verify control concentration before placing in-service.

12.5 Apparatus

12.5.1 Agilent GC/MSD, ChemStation software, compatible computer and printer.

12.5.2 Column: DB or HP-1 or HP-5 MS; 30 m x 0.25 mm x 0.25 µm.

12.5.3 Test tubes, 16 x 125 mm screw cap tubes, borosilicate glass with Teflon caps.

12.5.4 Test tubes, 16 x 125 mm round bottom tubes, borosilicate glass.

12.5.5 Test tubes, 16 x 114 mm (10 mL) glass tubes, conical bottom.

12.5.6 Centrifuge capable of 2,000 – 3,000 rpm.

12.5.7 Varian Bond Elute LRC Certify solid phase extraction (SPE) columns or United Chemical Technologies (UCT) SPE columns (ZSDAU020).

12.5.8 Solid phase extraction manifold.

12.5.9 Vortex mixer.

12.5.10 Evaporator/concentrator.

12.5.11 GC autosampler vials and inserts.

12.5.12 GCMS Instrument conditions. The following instrument conditions may be modified to adjust or improve separation and sensitivity.

12.5.12.1 Oven program

- Equilibration time: 0.50 minutes
- Initial temp: 160° C
- Initial time: 0 minutes
- Ramps:

	Final Temp(° C)	Final Time (minutes)
1. 35 °C / min	195	0
2. 5.0 °C / min	240	0
3. 30 °C / min	300	2.0

- Run time: 14 minutes

12.5.12.2 Inlet

- Mode: Splitless
- Temperature: 250° C
- Constant pressure: 13.88 psi
- Purge flow: 15.0 mL/min
- Purge time: 2.0 min
- Total flow: 18.9 mL/min
- Injection volume: 1.0 µL

12.5.12.3 MS Detector Temperature

- Transfer Line: 280° C
- Source: 230° C
- Quads: 150° C

12.5.12.4 Solvent delay: 10 minutes.

12.5.12.5 Acquisition mode: SIM (selected ion monitoring)

12.5.12.6 EM voltage: 200-600 over autotune depending on needed sensitivity

12.5.12.7 Dwell time: 40 milli-seconds

12.5.12.8 Ion Group 1

• Codeine	371	343	372
• Codeine d_3	374	346	
• Morphine	429	401	430
• Morphine d_3	432	417	

12.5.12.9 Ion Group 2

• Hydrocodone	386	371	387
• Hydrocodone d_3	389	374	
• 6-Acetylmorphine	399	340	400
• 6-Aceylmorphine d_3	402	343	
• Hydromorphone	444	355	429

- Hydromorphone d_3 447 358

12.5.12.10 Ion Group 3

- Oxycodone 474 459 475
- Oxycodone d_3 477 462
- Oxymorphone 532 517 533
- Oxymorphone d_3 535 520

12.6 Procedure (Two extraction procedures are presented as Option #1 and Option #2)

12.6.1 Extraction Option #1 (Varian Band Elute SPE columns)

12.6.1.1 Label 16 x 125 mm screw cap tubes accordingly, negative, calibrators, control (s) and case sample IDs.

12.6.1.2 Pipet 2 mL of each case sample into appropriately labeled tubes.

12.6.1.3 Pipet 100 µL of internal standard spiking stock to all tubes and vortex.

12.6.1.4 Add 6 mL of acetonitrile, cap and immediately shake each tube. Rotate tubes for 10 minutes.

12.6.1.5 Centrifuge tubes at approximately 2500 rpm for 5 minutes.

12.6.1.6 Decant supernatent into labeled 16 x 125 mm tubes.

12.6.1.7 Evaporate acetonitrile to 1-2 mL.

12.6.1.8 Add dH_2O to each tube to bring the total volume to approximately 3 mL.

12.6.1.9 Add 2 mL of pH 6.0, 0.1 M phosphate buffer to all tubes.

12.6.1.10 Add 0.5 mL of 1% hydroxylamine to each tube. Vortex briefly.

12.6.1.11 Place parafilm over the tops of tubes and incubate at 60 ^oC for one hour. Allow tubes to cool to room temperature.

12.6.1.12 Solid phase extraction (SPE). Place labeled Varian Bond Elute SPE cartridges in the extraction manifold.

12.6.1.12.1 Add 2 mL methanol and aspirate.

12.6.1.12.2 Add 2 mL pH 6.0 phosphate buffer and aspirate. Important! Do not permit SPE sorbent bed to dry. If necessary, add additional buffer to re-wet.

12.6.1.12.3 Pour specimens into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

12.6.1.12.4 Add 1 mL of 1 M acetic acid to each column and aspirate. Dry columns under full vacuum/pressure for at least 5 minutes.

12.6.1.12.5 Add 6 mL of methanol to each column and aspirate. Dry columns under full vacuum/pressure for at least 2 minutes.

12.6.1.12.6 Wipe the SPE column tips with Kimwipes®. Place labeled 10 mL conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

12.6.1.12.7 Add 2 mL of 2% ammonium hydroxide in ethyl acetate to each column. Collect eluant in conical test tubes by column aspiration or gravity drain.

12.6.1.13 Evaporate eluates to dryness.

12.6.1.14 Add 25 µL ethyl acetate and 25 µL BSTFA with 1% TCMS to each tube. Cap and vortex briefly.

12.6.1.15 Incubate for 20 minutes at 90° C.

12.6.1.16 Allow extracts to cool to room temperature.

12.6.1.17 Transfer derivatized extracts to autosampler vials.

12.6.2 Extraction Option #2 (UCT SPE columns)

12.6.2.1 Label 16 x 125 mm tubes accordingly, calibrators, controls and case sample ID's.

12.6.2.2 Prepare calibrators and controls.

12.6.2.3 Pipet 2 mL of each case sample into appropriately labeled tubes.

12.6.2.4 Pipet 100 µL of internal standard spiking stock to all tubes and vortex.

12.6.2.5 Add 3 mL cold (freezer temperature) acetonitrile dropwise to each tube, while votexing.

12.6.2.6 Centrifuge at 2800 rpm for 15 minutes.

12.6.2.7 Refrigerate overnight.

12.6.2.8 Transfer supernatants to clean test tubes.

12.6.2.9 Evaporate acetonitrile to 2 mL.

12.6.2.10 Add 2 mL of pH 6.0, 0.1 M phosphate buffer to tubes.

12.6.2.11 Add 0.5 mL of 1% hydroxylamine to each tube and vortex.

12.6.2.12 Cap tubes and incubate at 60° C for one hour. Allow tubes to cool to room temperature.

12.6.2.13 Solid Phase Extraction (SPE). Place labeled UCT SPE cartridges in the extraction manifold.

12.6.2.13.1 Add 3 mL hexane and aspirate.

12.6.2.13.2 Add 3 mL methanol and aspirate.

12.6.2.13.3 Add 3 mL dH_2O and aspirate.

12.6.2.13.4 Add 3 mL pH 6.0 phosphate buffer and aspirate. Important! Do not permit SPE sorbent bed to dry. If necessary, add additional buffer to re-wet.

12.6.2.13.5 Pour specimens into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

12.6.2.13.6 Add 1 mL of 1 M acetic acid to each column and aspirate.

12.6.2.13.7 Add 3 mL methanol and aspirate.

12.6.2.13.8 Dry columns under full vacuum/pressure (15 psi) for at least 10 minutes.

12.6.2.13.9 Wipe the SPE column tips with Kimwipes®. Place labeled 10 mL conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

12.6.2.13.10 Add 3 mL of 2% ammonium hydroxide in ethyl acetate (prepare fresh) to each column. Collect eluant in conical test tubes by column aspiration or gravity drain.

12.6.2.14 Evaporate eluates to dryness.

12.6.2.15 Add 30 µL ethyl acetate and 30 µL BSTFA with 1% TCMS to each tube. Cap and vortex briefly.

12.6.2.16 Incubate for 20 minutes at 90° C.

12.6.2.17 Allow extracts to cool to room temperature.

12.6.2.18 Transfer derivatized extracts to autosampler vials.

12.6.3 Extraction Option #3 (UCT SPE columns)

12.6.3.1 Label 16 x 125 mm tubes accordingly, calibrators, controls and case sample ID's.

12.6.3.2 Prepare calibrators and controls.

12.6.3.3 Pipet 2 mL of each case sample into appropriately labeled tubes.

12.6.3.4 Pipet 100 µL of internal standard spiking stock to all tubes and vortex.

12.6.3.5 Add 3 mL cold (freezer temperature) acetonitrile dropwise to each tube, while votexing.

12.6.3.6 Centrifuge at 2800 rpm for 15 minutes.

12.6.3.7 Refrigerate overnight.

12.6.3.8 Transfer supernatants to clean test tubes.

12.6.3.9 Evaporate acetonitrile to 2 mL.

12.6.3.10 Add 2 mL of pH 6.0, 0.1 M phosphate buffer to tubes.

12.6.3.11 Add 0.5 mL of 1% hydroxylamine to each tube and vortex.

12.6.3.12 Place parafilm over the tops of tubes and incubate at 60° C for one hour. Allow tubes to cool to room temperature. Place tubes in refrigerator while preparing SPE columns.

12.6.3.13 Solid Phase Extraction (SPE). Place labeled UCT SPE cartridges in the extraction manifold.

12.6.3.13.1 Add 3 mL methanol and aspirate.

12.6.3.13.2 Add 3 mL dH_2O and aspirate.

12.6.3.13.3 Add 3 mL pH 6.0 phosphate buffer and aspirate. Important! Do not permit SPE sorbent bed to dry. If necessary, add additional buffer to re-wet.

12.6.3.13.4 Pour specimens into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

12.6.3.13.5 Wash columns by adding 2 mL dH_2O and aspirate.

12.6.3.13.6 Add 2 mL 100 mM acetate buffer and aspirate.

12.6.3.13.7 Add 3 mL methanol and aspirate.

12.6.3.13.8 Dry columns under full vacuum/pressure (15 psi) for at least 10 minutes.

12.6.3.13.9 Wipe the SPE column tips with Kimwipes®. Place labeled 10 mL conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

12.6.3.13.10 Elute opiates by adding 3 mL 80/20/3 methylene choride/isopropanol/ammonium hydroxide to each column. Collect eluant in conical test tubes by column aspiration or gravity drain.

12.6.3.14 Evaporate eluates to dryness.

12.6.3.15 Add 25 µL ethyl acetate and 25 µL BSTFA with 1% TCMS to each tube. Cap and vortex briefly.

12.6.3.16 Incubate for 20 minutes at 90° C.

12.6.3.17 Allow extracts to cool to room temperature.

12.6.3.18 Transfer derivatized extracts to autosampler vials.

12.7 Calculation

12.7.1 Drug concentrations are calculated by linear regression analysis using the ChemStation software or off-line calculation.

12.8 Quality Control and Reporting

12.8.1 Report result to the nearest one hundredth (e.g. 0.01) according to Toxicology Quality Guidelines SOP for quality control and reporting.

12.8.2 6-acetylmorphine may be reported as “present”.

12.9 References

12.9.1 C.W. Jones, G. Chaney and S. Mastorides, “Simultaneous Analysis of Opiates in Urine by SPE and GC/MS with Stabilization of Keto-Opiates via Conversion to Oxime Derivatives.” Abstract #28, 1996 SOFT Annual Meeting, Denver, CO, ToxTalk Supplement, December 1996: 20(4): 9.

12.9.2 L.A. Broussard, L.C. Presley, T. Pittman, R. Clouette, and G.H. Wimbish. “Simultaneous Identification and Quantitation of Codeine, Morphine, Hydrocodone and Hydromorphone in Urine as Trimethylsilyl and Oxime Derivatives by Gas Chromatography-Mass Spectrometry,” Clin Chem 1997; 43: 1029-1032.

12.9.3 M.Cremese, A.H.B. Wu, G. Cassella, E. O'Connor, K. Rymut and D.W. Hill. "Improved GC/MS Analysis of Opiates with Use of Oxime-TMS Derivatives, " J. Forensic Sci 1998: 43(5): 1220-1224.

12.9.4 M.K. Lambing, G.D. Branum, J.D. Ropero-Miller and R.E. Winecker. "Simultaneous Quantification of Opioids in Blood by GC-EI-MS Analysis Following Deproteination, Detautomerization of Keto Analytes, Solid Phase Extraction and Silylated Derivatization," Abstract K12, American Academy of Forensic Sciences 2001: 312.

12.9.5 G.M. Vallaro and F. Mazur. "Confirmation of Opioids by GC/MS," UMASS Drug Concentration Laboratory SOP.

12.9.6 Varian Bond Elute Certify™ Instruction Manual